Bruton’s Tyrosine Kinase (BTK) Function is Important to the Development and Expansion of Chronic Lymphocytic Leukemia (CLL)

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Running Title: Importance of BTK function in CLL

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Key Points

- Kinase-functional BTK is important in the development and expansion of CLL
- Both targeted genetic inactivation of BTK and inhibition of BTK by ibrutinib inhibit the development of CLL in the TCL1 mouse model

Abstract

Chronic Lymphocytic Leukemia (CLL) demonstrates variable reactivity of the B cell receptor (BCR) to antigen ligation, but constitutive pathway activation. Bruton’s Tyrosine Kinase (BTK) shows constitutive activity in CLL, and is the target of irreversible inhibition by ibrutinib, an orally bioavailable kinase inhibitor that has shown outstanding activity in CLL. Early clinical results in CLL with other reversible and irreversible BTK inhibitors have been less promising, however, raising the question of whether BTK kinase activity is an important target of ibrutinib and also in CLL. To determine the role of BTK in CLL, we utilized patient samples and the Eμ-TCL1 (TCL1) transgenic mouse model of CLL which results in spontaneous leukemia development. Inhibition of BTK in primary human CLL cells by siRNA promotes apoptosis. Inhibition of BTK kinase activity through either targeted genetic inactivation or ibrutinib in the TCL1 mouse significantly delays the development of CLL, demonstrating that BTK is a critical kinase for CLL development and expansion and thus an important target of ibrutinib. Collectively, our data confirm the importance of kinase-functional BTK in CLL.
Introduction

CLL is a common adult leukemia that is currently incurable outside of stem cell transplantation. Though response to IgM ligation is variable, the B cell receptor (BCR) signaling pathway is aberrantly active in this disease, with antigen-dependent\textsuperscript{1, 2} or independent autonomous activation\textsuperscript{3} leading to constitutive activation of kinases inducing cell survival and proliferation.\textsuperscript{4-7} One BCR pathway kinase that is uniformly over-expressed at the transcript level\textsuperscript{8} and constitutively phosphorylated in CLL is Bruton’s tyrosine kinase (BTK). Ibrutinib, an orally bioavailable irreversible inhibitor of BTK, has recently been shown to have outstanding clinical activity in CLL with extended durable remissions in both untreated and relapsed disease.\textsuperscript{9}

BTK is a critical mediator of B lymphocyte signaling and development. Mutations in various domains are responsible for X-linked agammaglobulinemia (XLA),\textsuperscript{10, 11} a disorder characterized by developmental arrest of B cells and profound humoral immune deficiency in humans. A point mutation in the PH domain is responsible for the milder X-linked immunodeficiency (XID) phenotype in the mouse\textsuperscript{12, 13} which is characterized by reduced numbers of circulating B cells and reduced serum immunoglobulins. BTK is also a critical mediator in B cell signaling. It is recruited to the membrane-bound signalosome in the early stages of B cell activation, and, following phosphorylation by Syk and Lyn, participates in the phosphorylation of PLC\textsubscript{γ}2 which leads to production of the second messengers diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3). This pathway is amplified in CLL and leads to pro-survival signals through its effects on PI3K, PLC\textsubscript{γ}2, and NFkB.\textsuperscript{5, 8, 14, 15} Inhibition of BTK by ibrutinib interrupts BTK autophosphorylation after IgM ligation and reduces the expression of downstream targets of BCR activation including Erk, NF-κB, and Akt.\textsuperscript{8}

In addition to intracellular signaling, interaction of CLL cells with the microenvironment is controlled by BCR signaling and plays an important role in the survival and proliferation of
malignant cells in this disease.\textsuperscript{16, 17} Ibrutinib has been shown to inhibit microenvironment survival signals and block the protective effect of stromal co-culture in vitro.\textsuperscript{8}

It is apparent that BTK is critical for the development and function of normal B lymphocytes, and protein expression appears to be required for CLL development.\textsuperscript{18} However, the precise role of the kinase function of BTK in the initial development of CLL as well as the disease expansion phase is unclear. In addition, the concept of targeting a specific protein kinase in CLL, similar to targeting BCR-Abl in chronic myeloid leukemia, is one not generally believed to be feasible in CLL. Indeed, the lack of a ubiquitously amplified or mutated protein and overall heterogeneity of the disease suggests that multiple pathways would need to be targeted to achieve disease control. Ibrutinib covalently binds BTK at cysteine 481 within the hinge region and potentially cross-reacts with similar kinases which possess a homologous residue\textsuperscript{19} including some involved in B and T cell signaling such as BLK, TEC, and ITK.\textsuperscript{19} Ibrutinib's lack of selectivity raises the possibility that BTK is not the critical target in CLL and that alternative kinases or multiple kinases should be the focus of future drug development. Here we present a series of experiments using both primary CLL cells and the E\textsubscript{μ}-TCL1 transgenic mouse model of CLL. In this model, the TCL1 oncogene is under the control of the V\textsubscript{H} promoter-Ig\textsubscript{H}-E\textsubscript{μ} enhancer,\textsuperscript{20} which is first expressed in B cells at the transition to pre-B cells.\textsuperscript{21} Similar to what is observed in primary human CLL cells,\textsuperscript{8} in vitro cytotoxicity of ibrutinib in murine TCL1 leukemic spleen lymphocytes is modest, however, signaling through the BCR is inhibited \textit{in vitro} and \textit{in vivo}.\textsuperscript{22} The work outlined below will demonstrate that BTK is an important target in CLL and likely the critical kinase targeted by ibrutinib, and will thus validate BTK as a target for future drug development.
Materials and Methods

**CLL patient samples**

Blood samples were collected from patients that satisfied standard morphologic and immunophenotypic criteria for B cell CLL. Informed consent was obtained from all patients according to the Declaration of Helsinki and approval for the study was obtained from the institutional human research committee at the Catholic University Hospital "A. Gemelli". Mononuclear cells were isolated by Ficoll gradient centrifugation. CLL B cells were purified by negative selection with anti-CD3, anti-CD14 and anti-CD16 mouse monoclonal antibodies (kindly provided by Prof. Fabio Malavasi, University of Turin, Italy) and Dynabeads coated with pan anti-mouse IgG antibody (Invitrogen Dynal, Oslo, Norway). The purity of the selected B cell populations was determined by staining with anti-CD5 R-phycoerythrin (R-PE)–conjugated and anti-CD19 fluorescein (FITC)–conjugated antibodies (BD Biosciences, Franklin Lakes, NJ), followed by flow cytometry analysis on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). The purity of the negatively selected CLL B cell populations was >97%.

**siRNA nucleofection experiments**

For the BTK knockdown experiments, freshly isolated CLL cells were nucleofected with BTK Validated Stealth RNAi or Stealth RNAi Negative Control siRNA (Invitrogen, Carlsbad, CA). Nucleofections were performed on 6-8x10⁶ CLL cells using Nucleofector Solution V, the Amaxa Nucleofector II device and the U-013 program (Amaxa Biosystems GmbH, Cologne-Germany). Following nucleofection, CLL cells were resuspended at a cell density of 1x10⁷/mL in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate (Invitrogen, Carlsbad, CA). Cells were cultured alone, in the presence of immobilized anti-human IgM [2 x 10⁷/mL Dynabeads M-450 Epoxy (Invitrogen Dynal, Oslo, Norway) coated with 20 µg goat anti-human IgM (Southern Biotechnology Associates)] or in CLL-bone marrow stromal cell co-cultures.
[1x10^5/mL M2-10B4 bone marrow stromal cells (ATCC-LGC Standards, Middlesex, UK)], as described elsewhere. After 72 hours, the percentage of viable cells was determined by Annexin-A5-FITC conjugate/Propidium Iodide staining (Nexins Research, Kattendijke, The Netherlands) and flow cytometry. Downregulation of BTK was evaluated by immunoblotting analysis of cellular extracts obtained from aliquots of the same samples. Cellular extracts were separated by SDS-PAGE, transferred on Immobilon-P polyvinilidene difluoride membranes (Millipore, Billerica, MA) and blotted with BTK, rabbit IgG-HRP, mouse IgG HRP-linked (Cell Signaling Technology, Danvers, MA) or β-actin (Sigma-Aldrich, St Louis, MO) antibodies. Immunodetection and quantification were done on a Gel Logic 2200 Imaging System (Eastman Kodak, Rochester, NY), using ECL Plus enhanced-chemiluminescence detection reagents (Amersham Biosciences, Buckinghamshire, United Kingdom).

**Mice, mouse leukemia engraftment, and treatment with ibrutinib drinking water**

All animal experiments were performed under a protocol approved by the Ohio State University Institutional Laboratory Animal Care and Use Committee. TCL1 transgenic mice on C3H/C57BL/6(B6) background have been previously described. This mouse strain has been extensively back-crossed with the B6 mouse to obtain a TCL1 expressing mouse on a pure B6 background. Strain purity was confirmed by microsatellite analysis (Charles River laboratories, Wilmington, MA). To generate XID/TCL1 mice, homozygous B6/TCL1 mice were crossed with homozygous XID mice on a B6 background purchased from Jackson laboratories (Bar Harbor, ME). CB17/SCID mice for engraftments were purchased from Taconic Farms (Hudson, NY). Wild type B6 mice for engraftments were purchased from Jackson Laboratories.

For all engraftment studies, leukemic spleen lymphocytes were obtained from B6/TCL1 mice with high WBC counts and splenomegaly. Spleens were manually pulverized and spleen lymphocytes were purified by Ficoll density gradient separation. Flow cytometry was used to
confirm that lymphocytes were leukemic, and then spleen lymphocytes were resuspended in sterile phosphate-buffered saline and injected through a lateral tail vein. For SCID engraftments, $1\times10^6$ cells were injected into each mouse. For XID and B6 engraftments, $5\times10^6$-$5\times10^7$ cells were injected into each mouse, with equal amounts used for each individual experiment. Submandibular bleeding was used to obtain blood for smears and flow cytometry to evaluate disease progression. For experiments investigating survival, mice were euthanized for lethargy, difficulty walking due to spleen size, or loss of $\geq 20\%$ body weight. Animals were evaluated daily, with formal measurements of weight weekly during engraftment studies, and bi-weekly or monthly for long term studies.

For ibrutinib treatment experiments, mice were provided drinking water via water bottles containing either 10% β-cyclodextrin (vehicle) or vehicle plus 0.16 mg/mL ibrutinib. Mice were allowed to drink liberally, but were not provided another source of water. This drinking water formulation was kindly provided by Pharmacyclics, Inc. Mice receiving this as the only source of drinking water will receive approximately 30 mg/kg/day of drug (Supplemental Table 1).

**Mouse flow cytometry**

Flow cytometry to determine peripheral blood leukemia involvement was performed by staining fresh whole blood with anti-mouse CD45 APC, anti-mouse CD5 FITC, and anti-mouse CD19 PE (all from BD Biosciences, Franklin Lakes, NJ). CountBright beads were obtained from Invitrogen and used according to manufacturer’s instructions. Cells were initially doublet-discriminated, and then gated on the CD45 positive population. Flow cytometry was performed on a Beckman-Coulter FC-500 flow cytometer, and Kaluza software was used for data analysis.
Mouse immunoblot analysis

Whole cell lysates were prepared as previously described by our group.24 Equivalent amounts of protein were loaded into polyacrylamide gels and transferred onto nitrocellulose membranes. Following antibody incubations, proteins were detected with chemiluminescent substrate (SuperSignal, Pierce, Rockford, IL). pErk antibody was obtained from Millipore (Billerica, MA), Erk antibody was obtained from Cell Signaling Technology (Beverly, MA), and Actin antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Methods

Percent viability of CLL cells measured under different conditions are summarized with means and standard deviations and compared using paired t-tests. For the mouse models, estimates of leukemia-free survival, overall survival, and time to engraftment were obtained using the Kaplan-Meier method and the log-rank test evaluated differences between curves. Leukemia-free survival was defined as the time from birth or engraftment to the date of flow cytometry showing CD5/CD19 in ≥10% of lymphocytes. Overall survival was defined as the time from either birth or development of leukemia (noted in each individual experiment) until death. Time to engraftment was defined as the date of engraftment to the date of flow cytometry showing CD5/CD19 in ≥10% of lymphocytes. All tests were two-sided and statistical significance was declared for p<0.05.

Results

To first address the importance of BTK in CLL survival in vitro, we selectively down-regulated BTK protein by siRNA in primary tumor cells derived from 31 CLL patients (Figure 1A). Knock down of BTK results in a significant decrease in tumor cell survival as compared to control siRNA (p<0.001; Figure 1B). Induction of apoptosis was also seen in the presence of BCR stimulation or stromal protection (Figure 1C; Supplemental Figure 1), similar to what has been
observed with CLL cells treated with ibrutinib \textit{in vitro}.\textsuperscript{8} Collectively, these studies point toward the potential importance of this kinase in human CLL.

As we have seen that BTK knockdown leads to decreased CLL cell viability, we sought to determine whether the BTK kinase is a viable target in CLL \textit{in vivo}. To evaluate this we utilized the well characterized TCL1 mouse model which has been shown to possess genetic, epigenetic, and pharmacologic properties of human CLL.\textsuperscript{20, 25} B6/TCL1 mice were crossed with the well characterized XID mouse which has a point mutation in BTK preventing PH domain binding and therefore kinase activity.\textsuperscript{12, 13, 26} Cells derived from XID/TCL mice continue to express TCL1 protein (Figure 2A) and do not respond to IgM stimulation as robustly as WT/TCL1 mice (Supplemental Figure 2; Supplemental Figure 3). They have modestly decreased B cells and similar numbers of T cells compared to WT/TCL1 littermates (average 1843 B cells/\textmu L and 1379 T cells/\textmu L in XID/TCL1 vs 2157 B cells/\textmu L and 1236 T cells/\textmu L in WT/TCL1 mice). We next performed peripheral blood flow cytometry for CD5 and CD19 co-expressing leukemia cells on 59 WT/TCL1 and 61 XID/TCL1 mice of varying ages. At similar time points, the WT/TCL1 mice showed significantly higher percentage and number of leukemic cells than XID/TCL1 mice (Figure 2B). Furthermore, survival analysis of all male mice born within a 12 month period, which included 78 WT/TCL1 and 65 XID/TCL1, demonstrated superior survival in the XID/TCL1 cohort. Median overall survival (OS) for WT/TCL1 mice was 13.2 months (95% CI: 12.6 months - 14.0 months) versus 18.3 months for XID/TCL1 mice (95% CI: 15.4 months to not reached) (Figure 2C; \(p<0.0001\)). All XID/TCL1 mice that have been euthanized had flow cytometric evaluation of the spleen to detect leukemic cells. In a cohort of 49 mice which had spleen cells available for flow cytometry, 61\% had developed a CD5/CD19 co-expressing leukemia and 18\% had developed a T cell leukemia, which is also seen in the TCL1 mouse model. The delay in leukemia development and improvement in overall survival
thus demonstrate that kinase functional BTK is important for leukemia development in the TCL1 model of CLL, and suggest that BTK is therefore an important target of ibrutinib.

Next, we sought to determine whether ibrutinib is effective in the TCL1 mouse model, and whether ibrutinib could inhibit CLL development in a manner similar to genetic manipulation of BTK kinase activity. Prior studies with ibrutinib showing effects on disease progression and BCR signaling have utilized a serially transplanted TCL1 leukemia cell line model that was initially antigen selected to create consistent BCR expression.22 While this is an excellent tool for BCR signaling evaluation, it is not representative of the primary TCL1 leukemia cells which show more heterogeneous signaling (Supplemental Figure 4). To determine whether ibrutinib was also effective in spontaneous TCL1 leukemia, TCL1 leukemic spleen lymphocytes were transplanted into CB17/SCID mice. This model has the advantage of producing a CLL-like phenotype including peripheral blood leukemia, splenomegaly, and occasionally lymphadenopathy in a short period of time compared to the long latency period of the original TCL1 mouse model. At the time of leukemia diagnosis by flow cytometry, defined as ≥10% of lymphocytes co-expressing CD5 and CD19, mice were randomized to treatment with a drinking water containing 10% cyclodextrin (vehicle) or ibrutinib at a concentration providing approximately 30 mg/kg/day and which has been shown to occupy approximately 80% of available BTK in vivo (Supplemental Table 1, Supplemental Figure 5). BCR signaling, using phosphorylation of ERK as a surrogate, is inhibited in these mice (Supplemental Figure 2). From the time of leukemia diagnosis, mice treated with ibrutinib survived significantly longer (46 versus 24 days, p=0.003) with ibrutinib as compared to vehicle (Figure 3). Unlike human CLL, in this model no lymphocytosis was observed after treatment with ibrutinib. These data confirm that ibrutinib is effective in transplanted primary TCL1 leukemia cells not selected for enhanced BCR signaling and also that this drug inhibits the expansion phase of CLL in this model.
We next sought to determine whether inhibition of BTK kinase function by ibrutinib can prevent CLL development similar to that observed in XID/TCL1 mice outlined above. To evaluate this, TCL1 pups on both the B6 and C3H/B6 background were treated continuously starting at 1 month of age with ibrutinib or vehicle drinking water. Monthly assessment of CD5/CD19 leukemia cells by flow cytometry was performed for both groups. Mice treated with ibrutinib had a significantly prolonged time to leukemia development compared to vehicle (median 10.7 months [95% CI: 5.8 to 7.2] vs 7.0 months [95% CI: 10.1 to 13.0]; p<0.0001; Figure 4A). OS was also extended in mice treated with ibrutinib compared to vehicle (median 12.3 months [95% CI: 11.2 to 13.4] vs 14.5 months [95% CI: 14.1 to 15.6], p<0.0001; Figure 4B). This confirms that ibrutinib treatment inhibits the development of TCL1 leukemia similar to genetic inactivation of BTK.

**Discussion**

Collectively, our data show that kinase-functional BTK is critical to the development and expansion of CLL. While the inhibition in disease expansion demonstrated by the survival advantage in established disease is expected given the dramatic responses to this drug seen in the clinic, more remarkable is the significant delay in leukemia onset after targeted inactivation of BTK through either the XID/TCL1 model or the administration of ibrutinib. The XID/TCL1 cross definitively demonstrates that BTK is an important kinase in CLL, and the similar results seen with the BTK inhibitor ibrutinib suggest that this is the most relevant target of ibrutinib in CLL. While our data do not rule out the contribution of other related kinases to the efficacy of ibrutinib in patients, it does provide further support that BTK is indeed an important target of the drug.

The efficacy that has been seen with ibrutinib has led to preliminary development of more selective next-generation BTK inhibitors, but the discouraging preliminary results with more
selective inhibitors (AVL-292)\textsuperscript{27} questions whether ibrutinib is effective because it targets BTK, or because it has multiple alternative targets including ITK, TEC, and BLK.\textsuperscript{19} The dramatic leukemia inhibition seen with the XID/TCL1 cross shows that targeted inhibition of BTK kinase activity is sufficient to inhibit leukemia development in the TCL1 mouse model and suggests that clinical investigation of selective BTK inhibitors is reasonable.

This conclusion is important to our understanding of CLL, as ibrutinib therapy is the first instance where selective kinase inhibition is broadly effective in a disease without a uniform mutation or genetic modification. The model of selective kinase inhibition is effective with imatinib in chronic myeloid leukemia and vemurafenib in the subset of melanoma patients with the V600E BRAF mutation.\textsuperscript{28} Critically, however, in the studies that have examined recurrent DNA mutations in CLL, BTK has not been shown to be mutated in a significant subset of patients.\textsuperscript{29-31} These data therefore indicate that pathway activation, even in the absence of observed genetic modification, can serve as an effective therapeutic target in an otherwise genetically complex disease. BTK is an ideal candidate for selective inhibition as the severe phenotype of BTK dysfunction reveals its importance in B cells, however, the model may be applicable to other kinases in CLL and even other diseases.

It has recently been demonstrated that BTK expression is necessary for the development of leukemia in the IgH.ET\textsubscript{μ} mouse model,\textsuperscript{18} and it is notable that in this model where BTK was knocked out entirely, no leukemia was observed. In our model of the XID/TCL1 cross leukemia developed very late, but did still develop in the majority of animals. We believe that the data presented here are more applicable to human CLL, as drug development now and in the future will focus on inhibition of kinase function of BTK, rather than gene knockout. In addition, the TCL1 mouse model used in the experiments described here has been extensively characterized so that the similarities to human CLL are well established,\textsuperscript{20, 25} and the use of ibrutinib in this
model is directly relevant to human CLL. The fact that genetic knockout of BTK produced no disease, where disease is seen in the XID/TCL1 cross and the ibrutinib-treated TCL1 mice suggests that resistance to ibrutinib is mediated through BTK itself. In such case, the absence of BTK cannot be overcome, but drug inhibition, and even genetic mutation can. This finding is important to future work investigating mechanisms of resistance associated with ibrutinib.

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Conflicts of Interest: B.C. and J.J.B. are employees of Pharmacyclics. All other authors declare no relevant COI.
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Figure 1A: Knockdown of BTK by siRNA. Western blot analysis of BTK protein levels 72 hours after nucleofection with control or BTK-specific siRNA shows knock-down of BTK. This is representative of 37 patient samples.
**Figure 1B: CLL cell viability 72 hours after nucleofection with control or BTK siRNA.** Following BTK knockdown, cells were cultured for 72 hours, and viability determined at that time by percentage of cells negative for PI and Annexin V. Cells transfected with BTK siRNA had diminished viability compared to those treated with control siRNA.

![Graph showing viability of CLL cells](image)
Figure 1C: Viability of CLL cells after transfection with BTK or control siRNA and coincubation with stromal cells or stimulation with IgM. BTK knockout reduced CLL cell viability even in the presence of stromal cells or after IgM stimulation.
Figure 2A: TCL1 protein expression is retained after the cross with the XID mouse. Splenic lymphocytes from a pool of 3-1 month old female XID/TCL1 mice and 1 month old female B6/TCL1 mice were purified and B cell selected using the EasySep Mouse B Cell Enrichment Kit (Stem Cell Technologies) and then lysed. 50μg of protein from each mouse was used for Western Blot analysis of TCL1 protein expression. TCL1 is present in both the B6/TCL1 and the XID/TCL1 mice.
Figure 2B: WT/TCL1 mice have a higher percentage of leukemic lymphocytes in the peripheral blood compared with XID/TCL1 mice. Peripheral blood flow cytometry for CD5 and CD19 was performed on 59 WT/TCL1 and 61 XID/TCL1 mice. XID/TCL1 mice had a lower percentage of leukemic CD5/CD19 co-expressing cells than did WT/TCL1 mice.
Figure 2C: Overall survival is improved for XID/TCL1 mice compared with WT/TCL1 mice. All male XID/TCL1 and WT/TCL1 mice born within a 1 year time period, which included 65 XID/TCL1 and 78 WT/TCL1 mice were followed for survival. Overall survival is significantly prolonged in the XID/TCL1 cohort (p<0.0001).
Figure 3: Ibrutinib improves overall survival in mice transplanted with TCL1 leukemia. After transplantation with leukemic TCL1 spleen lymphocytes, SCID mice were followed for leukemia development, and once leukemic, randomized to treatment with ibrutinib or vehicle. Mice treated with ibrutinib (N=8) survived significantly longer than those treated with vehicle (N=9; p=0.003).
Figure 4A: Continuous administration of ibrutinib inhibits the development of CLL in TCL1 mice. TCL1 mice were randomized at 1 month of age to treatment with vehicle drinking water (n=24) or ibrutinib drinking water (n=24), and monitored for leukemia development by monthly peripheral blood flow cytometry. Ibrutinib significantly impairs leukemia development (p<0.0001).
Figure 4B: Continuous administration of ibrutinib improves survival in TCL1 mice. TCL1 mice were randomized at 1 month of age to treatment with vehicle drinking water (n=24) or ibrutinib drinking water (n=24), and followed for survival. Survival was significantly improved by ibrutinib administration as compared with vehicle (p<0.0001).
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